

# Antitumor Efficacy of *bcl-2* and *c-myc* Antisense Oligonucleotides in Combination with Cisplatin in Human Melanoma Xenografts: Relevance of the Administration Sequence

Gabriella Zupi,<sup>1</sup> Marco Scarsella,<sup>1</sup> Sean C. Semple,<sup>4</sup> Marcella Mottolose,<sup>2</sup> Pier G. Natali,<sup>3</sup> and Carlo Leonetti<sup>1</sup>

<sup>1</sup>Experimental Chemotherapy Laboratory, <sup>2</sup>Pathology Department, and <sup>3</sup>Immunology Laboratory, Regina Elena Cancer Institute, Rome, Italy; and <sup>4</sup>Inex Pharmaceuticals, Burnaby, British Columbia, Canada

## ABSTRACT

**Purpose:** *bcl-2* and *c-myc* oncogenes are frequently overexpressed in different human tumors, including melanoma. Here, we evaluate the combined efficacy of two antisense oligonucleotides targeting *bcl-2* mRNA (ODN *bcl-2*) and *c-myc* mRNA (ODN *c-myc*) in combination with cis-diammine dichloroplatinum (cisplatin, DDP) on three human melanoma lines (LM, NG, and M20).

**Experimental Design:** Two different sequences were designed to treat tumor-bearing mice: in the first one, ODN *bcl-2* at a dose of 0.2 mg/day × 4, followed by DDP given i.p. at a dose of 3.3 mg/kg/day × 3 and ODN *c-myc* i.v. at 0.5 mg/day × 7, whereas the other sequence consisted of ODN *c-myc* given as first agent followed by DDP and ODN *bcl-2* at the same doses. Mice received three complete cycles of treatment in 1-week intervals.

**Results:** The treatment sequence with ODN *bcl-2*/DDP/ODN *c-myc* combination completely inhibited growth in NG tumor and induced a 35-day delay in LM tumor growth. In contrast, the M20 tumor growth was unaffected by the combination. A discrete amount of c-Myc and *bcl-2* protein expression in both LM and NG tumors was detected, whereas no detectable levels of the two proteins were observed in M20 tumors. Compared with the other combination, the sequence ODN *bcl-2*/DDP/ODN *c-myc* produced the most effective results, producing a significant decrease in *bcl-2* and c-Myc protein expression, which in turn significantly increased the survival of NG- and LM-bearing mice, with 4 mice out of 11 and 1 out of 7 mice being cured, respectively. Finally, this combination increased the apoptotic rate and produced an antiangiogenic effect.

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**Requests for reprints:** Gabriella Zupi, Experimental Chemotherapy Laboratory, Regina Elena Cancer Institute, Via delle Messi d'Oro 156, 00158 Rome, Italy. Phone: 39-065266-2537; Fax: 39-065266-2592; E-mail: zupi@ifo.it.

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**Conclusions:** These results show that an antisense approach to the treatment of melanoma xenografts overexpressing either *bcl-2* or *c-myc* oncogenes represents a successful strategy to improve the response to chemotherapy in melanoma, with particular attention to the treatment sequence.

## INTRODUCTION

As increasingly evident, therapies based on the combination of conventional cytotoxic agents and targeted agents can result in an improvement of current cancer management. The possibility of impairing different molecular pathways that regulate tumor cell survival may be particularly advantageous, especially in the case of combinations free of side effects. Among the increasing number of target-specific drugs currently under investigation, antisense oligonucleotides (ODN) capable of interfering with tumor cell growth or apoptotic signaling as well as cancer cell invasiveness and metastatization, seem particularly promising for combination therapy in view of their lack of toxicity (1). Moreover, antisense strategies aimed at restoration of apoptotic signaling may be useful in overcoming tumor chemoresistance (2, 3).

Metastatic cutaneous melanoma is refractory to conventional therapy and current therapies are unable to significantly modify the prognosis of metastatic disease (4). Given that in the last decade, the incidence of this malignancy has risen to a rate equal or higher in comparison with the majority of all other human solid tumors (5), the development of new therapeutic compounds with preventive/therapeutic use is imperative. c-Myc overexpression is an independent negative prognostic marker for both primary (6) and metastatic melanoma (7). We have previously shown that the combination of cis-diammine dichloroplatinum (cisplatin, DDP) with c-myc antisense oligonucleotides (ODN *c-myc*), increases the cytotoxic activity of DDP by inhibiting cells to progress through the cell cycle. Furthermore, in melanoma xenografts, this treatment schedule resulted in a significant reduction in tumor mass and increased survival rates (8). In addition, we have shown that c-myc antisense oligonucleotides are able to overcome DDP resistance in a human melanoma cell line intrinsically resistant to this compound (3).

*bcl-2* expression in melanoma patients with regional lymph node metastasis is also associated with a significantly short survival rate (9). Experimental and clinical data have shown the efficacy of *bcl-2* antisense in combination with dacarbazine which is presently in phase III clinical trials (2, 10).

The aim of the present study was to verify the efficacy of the combined treatment of c-myc, *bcl-2* antisense oligonucleotides, and DDP in three melanoma xenografts differing in their sensitivity to DDP and displaying different expression of the two target proteins.

## MATERIALS AND METHODS

**Tumor Cell Lines and Xenografts.** The human melanoma lines used in this study characterized by different sensitivity to DDP (Table 1) were derived from a human primary melanoma (NG) and from metastatic lymph nodes (LM and M20) of three different patients undergoing surgery at the Regina Elena Cancer Institute (Rome, Italy). The tumor lines were obtained and maintained as previously described (3, 11, 12).

**Oligonucleotides and Drug.** A 16-mer antisense ODN (5'-TAACGTTGAGGGGCAT-3') complementary to the translation region of c-myc mRNA (ODN c-myc, INX-6295, Inex Pharmaceuticals, Corp., Burnaby, BC, Canada) was used. A scrambled ODN (scrambled ODN c-myc, INX-6300) containing the G-quartet motif (5'-TAAGCATACGGGGTGT-3') was used as a control sequence. An 18-mer antisense ODN (5'-TCTCCCAGCGTGCCCAT-3') complementary to the first six codons of bcl-2 mRNA (ODN bcl-2, oblimersen sodium; G3139, Genasense, Genta Incorporated, Berkeley Heights, NJ) was also employed. The reversed sequence (5'-TACCGCGTGCGACCCTCT-3') was used as a control (scrambled ODN bcl-2, G3622). All oligodeoxynucleotides were fully phosphorothioate. Clinical-grade DDP (Cisplatin Teva) was obtained from Teva Pharma (Netanya, Israel). DDP dilutions were freshly prepared before each experiment.

**In vivo Treatments.** Male CD-1 nude (*nu/nu*) mice (6-8 weeks old and 22-24 g in body weight) were purchased from Charles River Laboratories (Calco, Italy). Mice were injected in the hind leg with a cell suspension of  $2 \times 10^6$  cells. A tumor mass of about 300 mg was evident in all animals on day 6 after tumor implant and all treatments were started at this time. All procedures involving animals and their care were in accordance with institutional guidelines under the control of the Italian Ministry of Public Health. Each experimental group included 8 to 20 mice.

Percent tumor weight inhibition was evaluated at the nadir of the effect after three repeated cycles of DDP, which was administered for 3 consecutive days with 7-day intervals between each cycles, and calculated as  $[1 - (\text{mean tumor weight of treated mice} / \text{mean tumor weight of controls})] \times 100$ . The M20 tumor shows the highest sensitivity (tumor weight inhibition, 76%), with NG and LM tumors showing a tumor volume reduction of 35% and 20%, respectively. Moreover, no toxicity was observed in mice treated with DDP using this dosing schedule (Table 1).

Table 1 Origin and *in vivo* response to DDP of three different human melanoma cell lines

Lines*	Origin	% Tumor weight inhibition $\pm$ SD	Toxic deaths
NG	Primary tumor	35 $\pm$ 4	0/12
LM	Supraclavicular	20 $\pm$ 4	0/12
M20	metastatic lymph node	76 $\pm$ 13	0/12
	Supraclavicular metastatic lymph node		

\*Mice were injected i.p. with DDP at 3.3 mg/kg/days for 3 consecutive days starting from day 6 after tumor cell injection. Three cycles of treatment were administered at 7-day intervals. The percentage of tumor weight inhibition at the nadir of the effect was reported.

We then assessed the efficacy of DDP and ODN c-myc and ODN bcl-2 antisense in combination on the three tumor lines. To closely mimic the clinical setting, the two antisense oligonucleotides were administered i.v. The doses were selected based on previous results and preliminary experiments. ODN bcl-2 was injected i.v. at 0.2 mg/mouse/day, DDP was administered i.p. at 3.3 mg/kg/day, and ODN c-myc was injected i.v. at 0.5 mg/mouse/day. To compare the efficacy of the different treatment schedules, three cycles at 7-day intervals were administered as follows: schedule *a*, DDP alone, days 6 to 8, 16 to 18, and 26 to 28; schedule *b*, ODN c-myc  $\rightarrow$  DDP  $\rightarrow$  ODN c-myc, ODN c-myc at days 6 to 9, followed by DDP at days 10 to 12 and then followed by ODN c-myc at days 13 to 19; schedule *c*, ODN bcl-2  $\rightarrow$  DDP  $\rightarrow$  ODN bcl-2, ODN bcl-2 at days 6 to 9, followed by DDP at days 10 to 12 and then followed by ODN bcl-2 at days 13 to 19; schedule *d*, ODN bcl-2  $\rightarrow$  DDP  $\rightarrow$  ODN c-myc, ODN bcl-2 at days 6 to 9, followed by DDP at days 10 to 12 and then followed by ODN c-myc at days 13 to 19; schedule *e*, ODN c-myc  $\rightarrow$  DDP  $\rightarrow$  ODN bcl-2, ODN c-myc at days 6 to 9, followed by DDP at days 10 to 12 and then followed by ODN bcl-2 at days 13 to 19. A second and a third cycle of treatment were given at 7-day intervals. All the experiments were repeated at least thrice and representative independent experiments are reported.

**Western Blot Analysis.** To evaluate the expression level of c-Myc and bcl-2 protein in xenografts through Western blotting analysis, 100 mg of mechanically disaggregated control and treated tumors were solubilized in lysis buffer. Briefly, proteins (30  $\mu$ g) were separated by 10% SDS-PAGE, transferred to nitrocellulose filters, and incubated with monoclonal antibodies specific for human c-Myc (clone 9E10; Santa Cruz Biotechnology, Santa Cruz, CA) or for human bcl-2 (clone 124, DakoCytomation, Milan, Italy). After stripping, filters were incubated with anti-human  $\beta$ -actin antibody (clone JLA 20; Oncogene Science, Manhasset, NY), and reactivity was detected by enhanced chemiluminescence (Amersham International, Little Chalfont, Buckinghamshire, United Kingdom), according to the manufacturer's instructions. Results were quantified by scanning densitometry (Bio-Rad G700, Hercules, CA) of the autoradiography films and normalized to  $\beta$ -actin levels.

**Immunohistochemistry.** Expression of c-Myc and bcl-2 was analyzed immunohistochemically on formalin-fixed, paraffin-embedded sections of NG and LM tumor xenografts (two different experiments) using monoclonal antibody 9E11 (Novocastra, U.C.S., Rome, Italy), which was raised against a synthetic peptide representing amino acid residues 408 to 420 on the human c-Myc oncoprotein, and the bcl-2 monoclonal antibody 124 (DakoCytomation), respectively. The monoclonal antibody to Ki-67 (MIB-1, DakoCytomation) was used to identify the proliferation index (PI) of untreated and treated tumor cells. Antigen retrieving was achieved by pretreating dewaxed sections in a microwave oven at 750 W for 5 minutes in citrate buffer (pH 6), and then processing them with a Super Sensitive Link-Labeled Detection System (BioGenex, Menarini, Florence, Italy). The enzymatic activity was developed using 3-amino-9-ethylcarbazole (DakoCytomation) as a chromogenic substrate. Following counterstaining with Mayer's hematoxylin, slides were mounted in aqueous mounting medium (glycergel,

DakoCytomation). PI in LM and NG tumor cells was determined counting positive nuclei in eight high-power fields (400× magnification) per section. Negative controls consisted of parallel sections in which the primary antibody was omitted. Slides were observed by two independent observers with no knowledge of the experimental plan. When frozen samples were used (CD31 vessels stain), glass slides containing at least four nonconsecutive 4 sections were obtained which were air-dried and fixed for 10 minutes in absolute acetone. The CD31 murine monoclonal was obtained from Dr. A. Mantovani (Mario Negri Institute, Milan, Italy). The immune reactions was detected using the mouse on mouse kit obtained from Vector Labor (Burlingame, CA). The chromogen and nuclear counterstain were done as described above. Microvessel density was scored by evaluating at least 6 random fields of 10 nonconsecutive sections at ×20 magnification. Tumors from two animals from each treatment group was analyzed.

**Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling Assay.** The fluorescent *in situ* detection of apoptosis in formalin-fixed, paraffin-embedded tumor tissues (LM and NG) was done by terminal deoxynucleotidyl transferase-mediated nick end labeling (Enzo Life Sciences, DakoCytomation). Following deparaffinization and rehydration, 50 μL of terminal deoxynucleotidyl transferase-mediated nick end labeling reaction mixture were applied to the sections, and the slides were incubated at 37°C for 60 minutes. The slides were then washed thrice in PBS for 5 minutes, and the sections were examined using a fluorescence microscope. Following terminal deoxynucleotidyl transferase-mediated nick end labeling staining, sections were scanned at 200× magnification to identify areas with the greatest apoptosis. Apoptosis of LM and NG tumor cells were counted in eight high-power fields (400× magnification) per section. Two independent observers did the counts in blinded fashion. For each tumor, counts were averaged to determine the number of apoptotic cells.

**Statistical Analysis.** Survival curves were generated by Kaplan-Maier product-limit estimate, and statistical differences between the various groups were evaluated by log-rank analysis with Yates correction. The statistical difference of tumor weight and microvessel density among the different groups was determined by Student's *t* test assuming unequal variances. To determine the significant differences between group means in an ANOVA setting (AI/apoptotic index, PI/proliferation index, AI/PI ratio) the Bonferroni test was used. All proliferation and apoptosis results are expressed as a median percentage. Differences were considered statistically significant when  $P < 0.05$ .

## RESULTS

**Antitumor Activity of the Combination of bcl-2 and c-myc Antisense Oligonucleotides and Cisplatin.** Figure 1 shows the tumor growth of LM (A) NG (B) and M20 (C) xenografts treated with the different combination schedules. DDP alone inhibited LM tumor growth by 20%, which was not significantly different from the untreated tumors. The three administration sequences, ODN bcl-2→DDP→ODN bcl-2, ODN c-myc→DDP→ODN c-myc, and ODN c-myc→DDP→ODN bcl-2 inhibited tumor growth by 49%, 60%, and

50%, respectively; interestingly, the combination ODN bcl-2→DDP→ODN c-myc resulted in a tumor weight inhibition of 91% and a tumor growth delay of 35 days (Fig. 1A). NG tumors showed a greater sensitivity to DDP than the LM tumors, with a 35% reduction in tumor growth, whereas the sequence ODN bcl-2→DDP→ODN bcl-2 caused a 68% tumor inhibition, the sequence ODN c-myc→DDP→ODN c-myc resulted in a 78% tumor inhibition, and the sequence ODN c-myc→DDP→ODN bcl-2 inhibited tumor growth by 65%. In NG tumors, on the other hand, the sequence ODN bcl-2→DDP→ODN c-myc caused an inhibition of tumor weight of 95% in ~60% of mice; a complete regression of tumor was observed in the other mice, which were not included in the tumor growth curve (Fig. 1B).

On the contrary, the M20 tumor, which is relatively more sensitive to DDP, was unaffected by the combined treatment (Fig. 1C). In fact, similar tumor weight inhibition was observed in treated mice with DDP alone or with each of the three combinations (range, 76-81% tumor weight inhibition). The combined therapy was probably unable to modulate sensitivity to DDP treatment due to the fast growth rate of M20, which simulates exponential growth. In fact, 20 days after tumor implantation, a tumor mass of ~4,000 mg was already evident. The administration of bcl-2 and c-myc scrambled ODN in NG melanoma bearing animals was ineffective (Fig. 1D). Similar results were obtained with LM tumors (data not shown).

**c-Myc and bcl-2 Expression and their Down-regulation After Treatments.** The ineffectiveness of the combined treatment on M20 tumors led us to analyze the *in vivo* expression of c-Myc and bcl-2 proteins on the three tumors xenografts. Figure 2A shows c-Myc and bcl-2 protein expression in untreated LM, NG, and M20 tumors excised from three different mice when a tumor mass of about 300 mg was evident. Densitometric analysis of Western blots showed that NG tumors displayed about 40% less c-Myc and 20% less bcl-2 compared with LM tumors. c-Myc and bcl-2 were undetectable in M20 xenografts.

These results were surprising because human melanoma has been reported to express high levels of c-Myc and bcl-2, but explain why the combined treatment was ineffective. We then analyzed the expression level of c-Myc and bcl-2 4 days after the end of the first cycle of treatment (Fig. 2B). DDP used as single agent did not decrease c-Myc expression in LM tumors, whereas a near 30% decrease in c-Myc protein level was obtained in NG tumors. c-Myc protein expression decreased in LM and NG tumors when the mice were treated with the sequence ODN c-myc→DDP→ODN c-myc, the greater decrease being noted in NG tumors. No c-Myc expression was detected in either tumor lines treated with the combination ODN bcl-2→DDP→ODN c-myc. Similar results were observed for bcl-2 expression, a higher bcl-2 down-regulation being observed in NG tumors compared with LM tumors. Also, bcl-2 protein level was undetectable administering the combination ODN bcl-2→DDP→ODN c-myc. It is interesting to note that when LM- or NG-bearing mice were treated with the schedule ODN c-myc→DDP→ODN c-myc, in addition to the marked decrease in c-Myc protein, a reduction in bcl-2 protein expression was also observed; this is true also for the

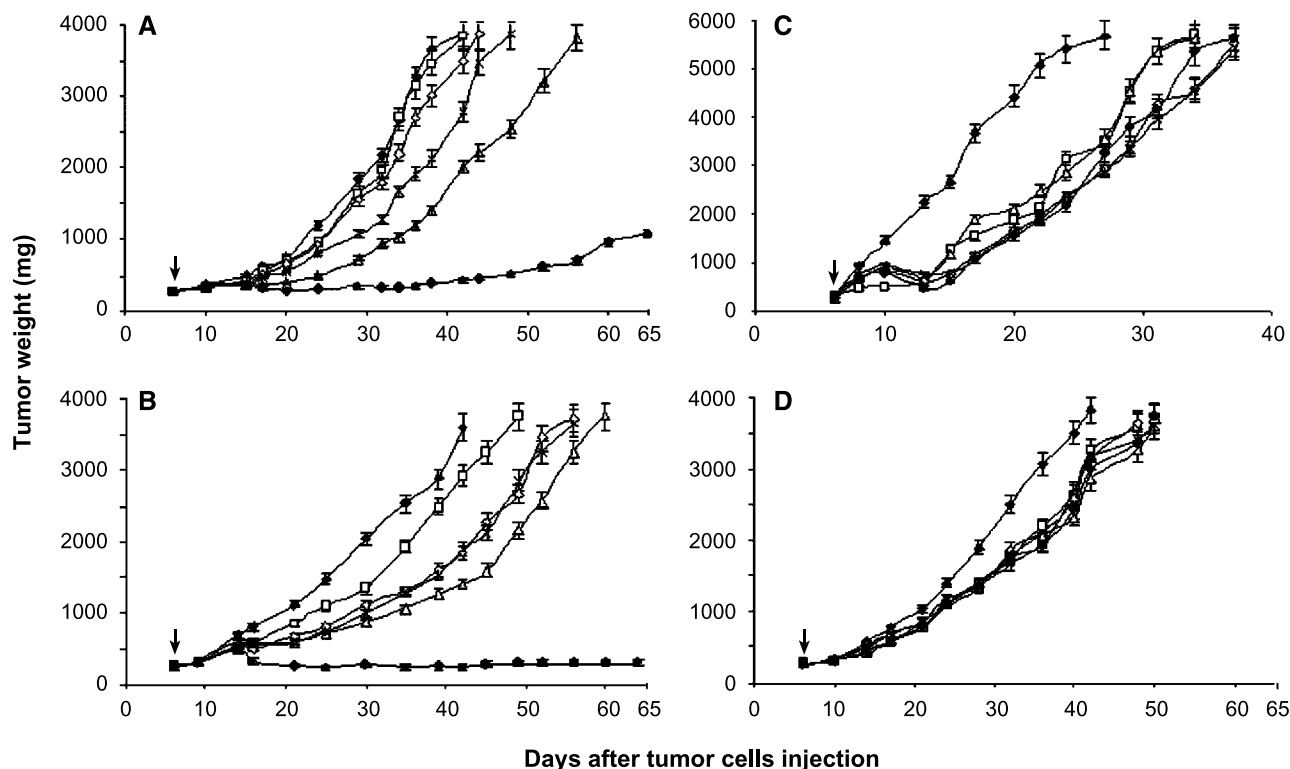
combination ODN bcl-2→DDP→ODN bcl-2 which also reduced the expression of c-Myc protein, even though to a lesser extent than bcl-2 protein expression. These results were confirmed by immunohistochemistry analysis, where >80% of tumor cell nuclei displayed c-Myc expression and a strong and homogeneous cytoplasmic positivity to bcl-2 protein was evident in untreated NG and LM tumors, while a lack of immunostaining was evident in tumors from mice treated with the schedule ODN bcl-2→DDP→ODN c-myc (data not shown).

c-Myc and bcl-2 protein levels found in lysates from tumors of mice treated with DDP and scrambled ODN in combination were superimposable with levels of untreated or DDP-treated mice (Fig. 2C).

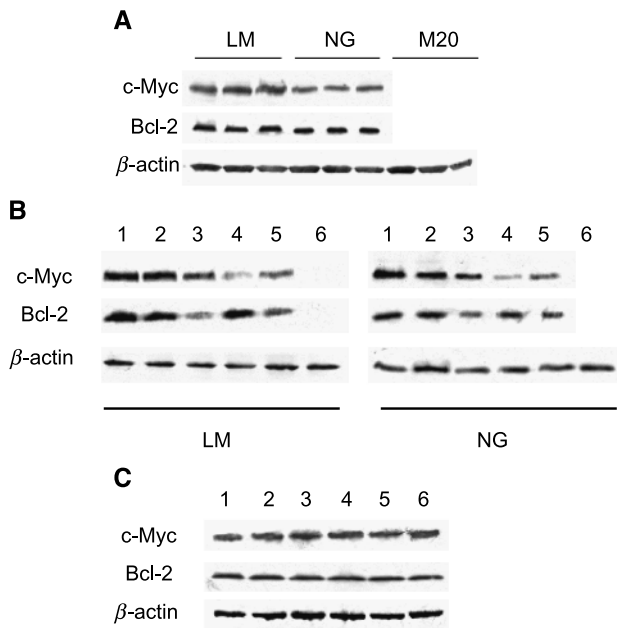
**Combination Treatment Results in an Increased Survival Rate in Mice.** Figure 3 shows the survival curves of mice bearing LM (A) and NG (B) tumors treated with the different combinations. The treatments gave consistent results in different experiments with a significant increase in life span of mice which received the combination ODN bcl-2→DDP→ODN

c-myc. In fact, mice bearing LM tumors and treated with this combination showed a mean survival of 177 days compared with 76 days of the control mice (Fig. 3A) and with one out of seven animals being tumor-free 12 months after the end of the experiments. As expected from the tumor growth, the statistical analysis shows that only the sequence ODN c-myc→DDP→ODN c-myc and ODN bcl-2→DDP→ODN c-myc showed significant differences from the control group. Moreover, the sequence ODN bcl-2→DDP→ODN c-myc is statistically different from the sequence ODN c-myc→DDP→ODN c-myc ( $P = 0.023$ ).

Similar results were obtained in mice bearing NG tumors (Fig. 3B). Each of the experimental groups were statistically different from the control group. The administration of the combination ODN bcl-2→DDP→ODN c-myc shows a significant difference compared with the other three sequence combinations ( $P < 0.001$ ), with 139-day mean survival compared with the 72 days of the control animals. Moreover, 4 mice out of 11 were cured using the combination ODN bcl-2→DDP→ODN



**Fig. 1** Antitumor activity of DDP alone or in combination with ODNs on human melanoma-bearing mice. Mice were implanted i.m. with LM (A), NG (B and D), or M20 (C) melanoma cells and treated for three cycles of treatment at 7-day intervals, according to the following schedules: A-C, (●) untreated; (□) DDP (schedule a); (△), ODN c-myc→DDP→ODN c-myc (schedule b); (◇), ODN bcl-2→DDP→ODN bcl-2 (schedule c); (●), ODN bcl-2→DDP→ODN c-myc (schedule d); (\*), ODN c-myc→DDP→ODN bcl-2 (schedule e). D, (●) untreated; (□) DDP; (△), scrambled ODN c-myc→DDP→scrambled ODN c-myc; (◇), scrambled ODN bcl-2→DDP→scrambled ODN bcl-2; (●), scrambled ODN bcl-2→DDP→scrambled ODN c-myc; (\*), scrambled ODN c-myc→DDP→scrambled ODN bcl-2; ODN bcl-2 or scrambled ODN bcl-2 were administered i.v. at 0.2 mg/mouse/day for 4 days, DDP was administered i.p. at 3.3 mg/kg/day for 3 days and ODN c-myc or scrambled ODN c-myc were administered i.v. at 0.5 mg/mouse/day for 7 days. Mean tumor weight, mg  $\pm$  SD; arrows, start of treatments. A, schedule d is significantly different from untreated and all treated groups ( $P < 0.001$ ), except for schedule e ( $P > 0.05$ ). Schedules a, c, and e are not significantly different from each other and from untreated; B, schedule d is significantly different from untreated and all treated groups ( $P < 0.001$ ). Schedule b is significantly different from schedule a ( $P < 0.001$ ). All the schedules are significantly different from untreated ( $P < 0.001$ ) except for schedule a ( $P > 0.05$ ). Schedules b, c, and e are not significantly different from each other ( $P > 0.05$ ). Schedules c, e, and a are not significantly different from each other; C, all treated groups are statistically different ( $P < 0.001$ ) versus untreated group; D, no statistical significance ( $P > 0.05$ ) was observed between treated and untreated groups.



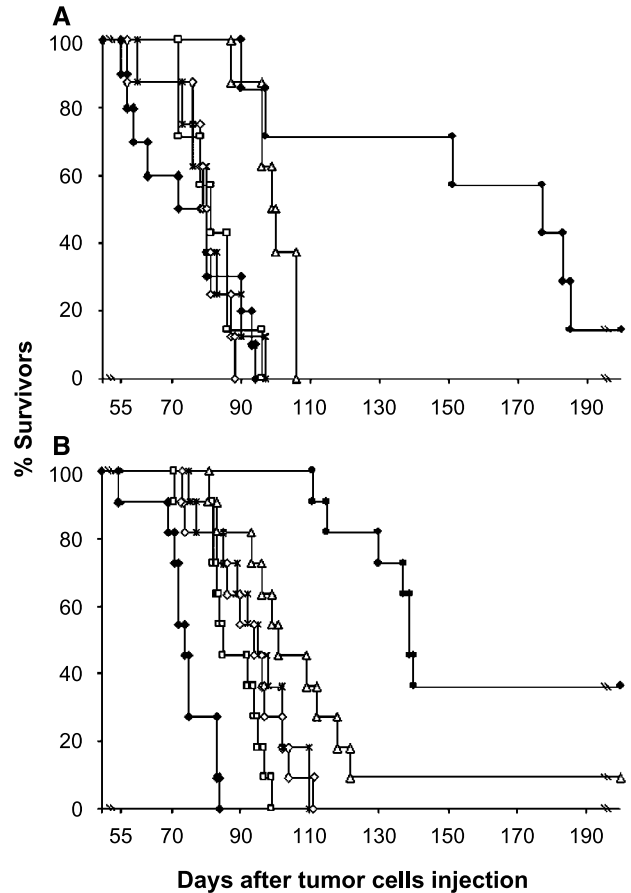
**Fig. 2** Western blot analysis of c-Myc and Bcl-2 protein in untreated or melanoma treated with DDP alone or in combination with ODNs. *A*, tumors were excised from untreated mice when a tumor mass of about 300 mg was evident. Three different mice for each melanoma line were analyzed. *B*, LM and NG tumors were excised from mice at day 4 after the end of the first cycle of treatment. Lane 1, untreated; lane 2, DDP; lane 3, ODN bcl-2→DDP→ODN bcl-2; lane 4, ODN c-myc→DDP→ODN c-myc; lane 5, ODN c-myc→DDP→ODN bcl-2; lane 6, ODN bcl-2→DDP→ODN c-myc. The expression of c-Myc and bcl-2 protein was quantified and normalized to the corresponding β-actin protein amount. Percentage of c-Myc or bcl-2 protein inhibition in treated versus untreated groups is reported. (*Left, c-Myc*) lane 2, 14%; lane 3, 32%; lane 4, 76%; lane 5, 58%; lane 6, 96%; (*bcl-2*) lane 2, 8%; lane 3, 70%; lane 4, 32%; lane 5, 60%; lane 6, 98%. (*Right, c-Myc*) lane 2, 30%; lane 3, 58%; lane 4, 80%; lane 5, 60%; lane 6, 98%; (*bcl-2*) lane 2, 18%; lane 3, 64%; lane 4, 34%; lane 5, 54%; lane 6, 98%. *C*, NG tumors were excised from mice at day 4 after the end of the first cycle of treatment. Lane 1, untreated; lane 2, DDP; lane 3, scrambled ODN bcl-2→DDP→scrambled ODN bcl-2; lane 4, scrambled ODN c-myc→DDP→scrambled ODN c-myc; lane 5, scrambled ODN c-myc→DDP→scrambled ODN bcl-2; lane 6, scrambled ODN bcl-2→DDP→scrambled ODN c-myc.

c-myc. These results show that the sequence of administration plays a crucial role in the improvement of mice survival.

**Combination Treatment Induces Apoptosis and Reduces the Proliferation Index.** By terminal deoxynucleotidyl transferase-mediated nick end labeling assay, we analyzed the ability of different treatments to trigger apoptosis in LM and NG melanoma xenografts. The combination ODN bcl-2→DDP→ODN c-myc was able to markedly increase the extent of AI in LM tumors (Fig. 4A), with cell death significantly more extensive than the three combination treatments ( $P < 0.001$ ). In NG tumors (Fig. 4D), the sequence ODN bcl-2→DDP→ODN c-myc showed the highest AI compared with untreated and to all treated groups ( $P < 0.001$ ).

The PI of LM and NG tumors was also evaluated. Tumors from untreated mice presented a significantly higher PI (80%) than mice treated with DDP alone or with the different combination in both LM (B) and NG (E) tumors.

Again, ODN bcl-2→DDP→ODN c-myc was significantly more effective in reducing proliferation compared with all other treatment groups with a ( $P < 0.001$ ). The AI/PI ratio revealed that it was significantly increased ( $P < 0.001$ ) compared with controls and other treatments only in ODN bcl-2→DDP→ODN c-myc-treated mice, with values being higher in NG tumors ( $1.28 \pm 0.3$  SD, *F*) than LM tumors ( $0.91 \pm 0.38$  SD, *C*).



**Fig. 3** Survival of mice bearing human melanoma untreated or treated with DDP alone or in combination with ODN bcl-2 and/or ODN c-myc. Mice were implanted i.m. with LM (A) or NG (B) melanoma and treated as reported in Fig. 1, according to the following schedules: (◆) untreated; (□) DDP (schedule a); (△), ODN c-myc→DDP→ODN c-myc (schedule b); (◇), ODN bcl-2→DDP→ODN bcl-2 (schedule c); (●), ODN bcl-2→DDP→ODN c-myc (schedule d); (\*), ODN c-myc→DDP→ODN bcl-2 (schedule e). Survival of mice treated with the different schedules were compared, as reported in Materials and Methods, to evaluate statistical significance of the differences. *A*, schedule d is significantly different from untreated and from all treated groups ( $P < 0.001$ ), except for schedule b ( $P = 0.023$ ). Schedule b is significantly different from untreated and from all treated groups ( $P < 0.001$ ). Schedules a, c, and e are not significantly different from each other and from untreated; *B*, schedule d is significantly different from untreated and from all treated groups ( $P < 0.001$ ). Schedule b is significantly different from untreated group ( $P < 0.001$ ) and from schedules a, c, and e ( $P = 0.0032$ ,  $P = 0.043$ , and  $P = 0.02$ ), respectively. Schedule a is significantly different from untreated group ( $P = 0.012$ ). Schedules c and e are significantly different from untreated group ( $P < 0.001$ ). Schedules a, c, and e are not significantly different from each other.

Figure 5A and B report representative findings of apoptosis in untreated and tumors treated with the combination ODN bcl-2→DDP→ODN c-myc in NG tumors.

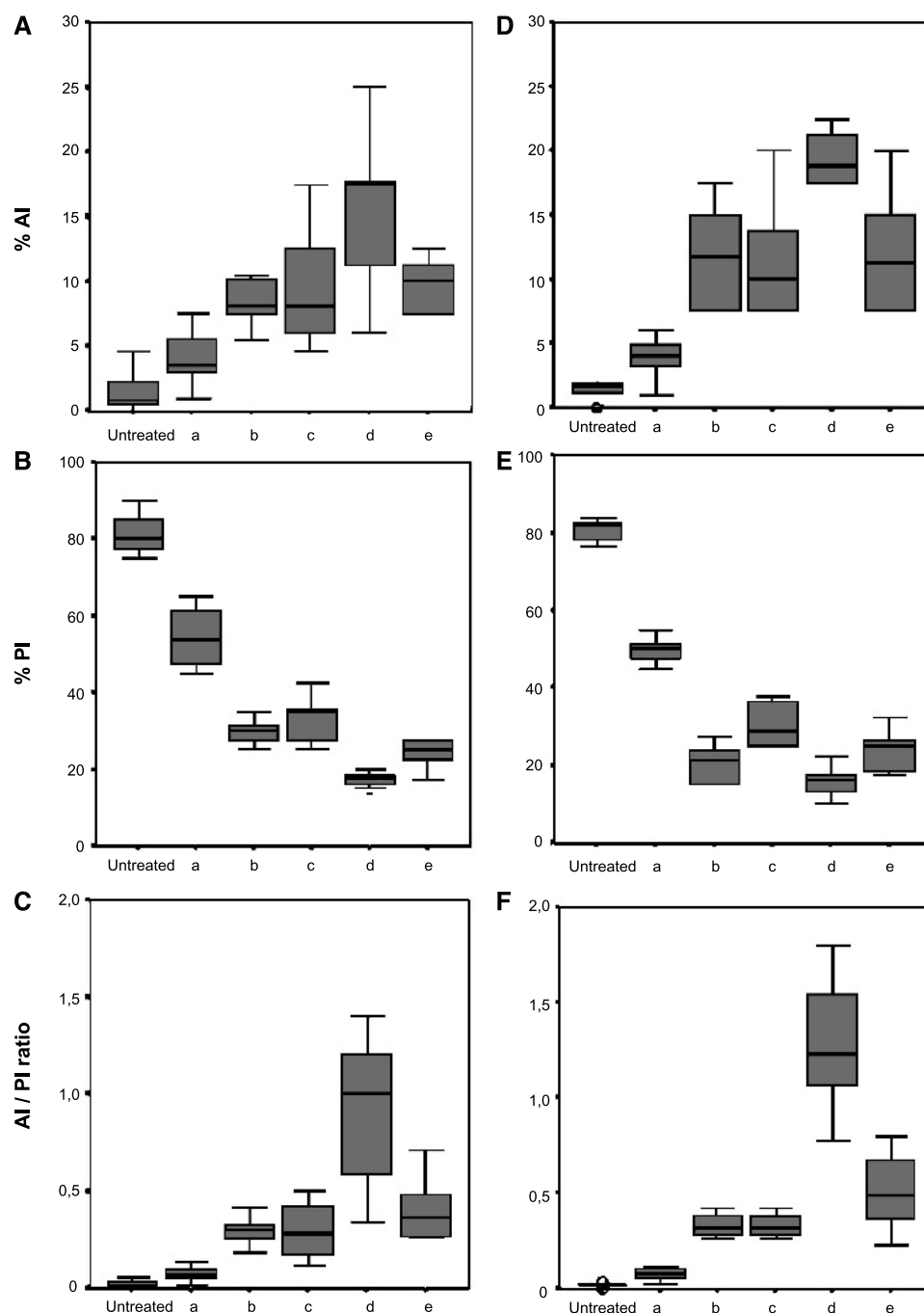
**Combination Treatment Reduces Vessel Formation.** Table 2 reports the results of the microvessel density, demonstrating a statistically significant decrease in all the tumors treated with the different combinations compared with untreated and DDP-treated tumors. The highest reduction in microvessel density was observed in LM and NG tumors treated with the most effective sequence ODN bcl-2→DDP→ODN c-myc. The statistical analysis reported

in the table showed that this sequence is significantly different from the other sequences employed for both LM and NG tumors. These findings are similar in LM and in NG tumors. Representative findings of this analysis are reported in Fig. 5C and D.

## DISCUSSION

The outcome of advanced cutaneous melanoma remains poor despite the continuous testing of novel biological, chemotherapeutic, and combined treatments. In this study, we show that combined treatment of DDP with antisense

*Fig. 4* AI and PI in NG and LM tumors untreated or treated with DDP alone or in combination with ODN bcl-2 and/or ODN c-myc. LM tumors (A-C) and NG tumors (D-F) were treated according to the following schedules: untreated; DDP (schedule a); ODN c-myc→DDP→ODN c-myc (schedule b); ODN bcl-2→DDP→ODN bcl-2 (schedule c); ODN bcl-2→DDP→ODN c-myc (schedule d); ODN c-myc→DDP→ODN bcl-2 (schedule e). Tumors were excised from mice at day 4 after the end of the first cycle of treatment. Results shown are as follows: AI (A and D), PI (B and E) and AI/PI ratio (C and F). Lines, median; Boxes, 25th and 75th percentiles; whiskers, minimum and maximum values. Statistical significance of differences of AI/PI ratio between each group, evaluated as reported in Materials and Methods, are as follows: C (LM tumor), schedule d is significantly different from untreated and from all treated groups ( $P < 0.001$ ). Schedules a, b, c, and e are not significantly different from each other and from the untreated group. F (NG tumor), schedule d is significantly different from untreated and from all treated groups ( $P < 0.001$ ). All the schedules are significantly different from untreated group ( $P < 0.05$ ) except for schedule a ( $P > 0.05$ ). Schedules b, c, and e are significantly different from schedule a ( $P < 0.05$ ). Schedules b, c, and e are not significantly different from each other.



**Table 2** Changes in microvessel densities in LM and NG tumors from mice untreated or treated with DDP alone or in combination with ODN c-myc and/or ODN bcl-2

Scheduling	LM	NG
Untreated	49 ± 7*	38 ± 4
(a) DDP	47 ± 8	36 ± 3
(b) ODN c-myc→DDP→ODN c-myc	31 ± 4	21 ± 3
(c) ODN bcl-2→DDP→ODN bcl-2	28 ± 5	30 ± 5
(d) ODN bcl-2→DDP→ODN c-myc	22 ± 2	12 ± 4
(e) ODN c-myc→DDP→ODN bcl-2	30 ± 5	25 ± 6

NOTE. Statistical analysis: LM, schedule *d* is significantly different from untreated and all treated groups ( $P < 0.001$ ) except for schedule *b* ( $P = 0.021$ ). All the schedules are significantly different from untreated group ( $P < 0.001$ ) except for schedule *a* ( $P > 0.05$ ). Schedules *b*, *c*, and *e* are significantly different from schedule *a* ( $P < 0.001$ ) but are not significantly different between each other. NG, schedule *d* is significantly different from untreated and all treated groups ( $P < 0.001$ ). All the schedules are significantly different from untreated ( $P < 0.001$ ) except for schedule *a* ( $P < 0.05$ ). All the schedules are significantly different from schedule *a* ( $P < 0.001$ ) except for schedule *c* ( $P > 0.05$ ). Schedule *b* is significantly different from schedule *c* ( $P < 0.05$ ), but not from schedule *e* ( $P > 0.05$ ).

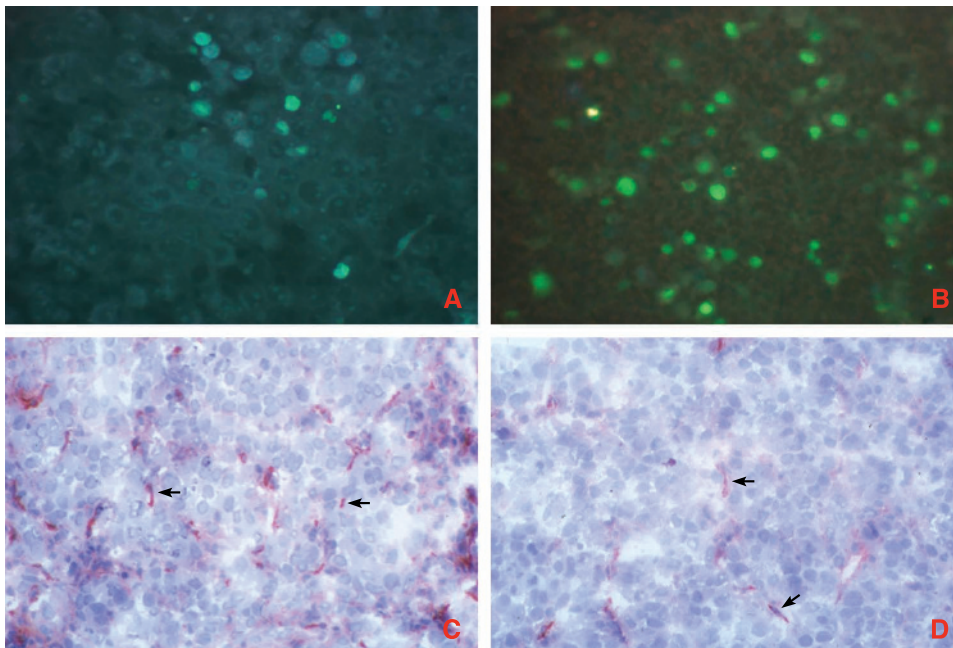
\*Mean number of microvessels ± SD. Tumors were excised from mice at day 4 after the end of the first cycle of treatment.

ODN targeting c-Myc and bcl-2 according to the sequence ODN bcl-2→DDP→ODN c-myc for three cycles is effective in controlling the growth of established (300 mg) melanoma xenografts. This sequence of administration shows an enhanced therapeutic efficacy compared with reverse sequence ODN c-myc→DDP→ODN bcl-2, consistent with previous findings which showed that the use of ODN bcl-2 as the first drug sensitizes to chemotherapy (2) and that ODN c-myc administered after DDP prevents cells from progressing through the cell cycle (8). These data show the relevance of the sequence of administration in improving the antitumor efficacy.

The efficacy of the combination ODN bcl-2→DDP→ODN c-myc correlates with the level of expression of the two target genes in melanoma cells; the treatment, in fact, was ineffective in M20 tumors which lack detectable levels of c-Myc and bcl-2 proteins. On the contrary, the combination treatment was effective both in NG and LM tumors which express high levels of c-Myc and bcl-2. Moreover, NG tumors are more sensitive to ODN bcl-2→DDP→ODN c-myc compared with the LM tumors. This might be due to the increased sensitivity to DDP of NG melanoma. Moreover, in NG tumors, treatment with DDP reduces the proliferation and decreases the expression of the two proteins. On the contrary, no down-regulation of c-Myc and bcl-2 proteins was observed in DDP-treated LM tumors. The effect of DDP in reducing both c-myc and bcl-2 expression might contribute to the increased efficacy of ODN bcl-2→DDP→ODN c-myc combination in NG tumors.

All these results clearly show that two antisense oligonucleotides targeted to different genes cooperate to increase the efficacy of DDP, even in the case of relatively drug-resistant tumors such as LM tumors.

The present findings are in agreement with reports demonstrating that bcl-2 antisense oligonucleotides sensitize melanoma cells to several antineoplastic drugs and synergize with DDP in human lung cancer cell lines (13). Moreover, combined treatment with bcl-2 antisense and DDP prolongs survival of the human gastric cancer-bearing severe combined immunodeficiency mice (14). Furthermore, bcl-2 down-regulation obtained with small interfering RNA, when combined with DDP, causes a massive increase in apoptotic cell death determining a supra-additive effect (15). The available data demonstrating the ability of bcl-2 antisense in down-regulating the bcl-2 protein and in chemosensitizing tumors of different histotypes (16–18), should be interpreted with caution in view of the inherent nonspecificity of phosphorotioate oligonucleotides,



**Fig. 5** Detection of apoptosis (*A* and *B*) by terminal deoxynucleotidyl transferase-mediated nick end labeling (apoptotic cells appear fluorescent green) and angiogenesis (*C* and *D*) by immunostaining of microvessels by anti-CD31 (arrows) in NG tumors excised from mice at day 4 after the end of the first cycle of treatment. Sections shown are as follows: untreated (*A* and *C*) and ODN bcl-2→DDP→ODN c-myc (*B* and *D*). Original magnification, ×250.

including bcl-2 antisense (19–21). Moreover, additional “non-specific” mechanisms contribute to the antiproliferative effects of bcl-2 antisense which are likely to depend on the presence of “bis-CpG” motif in their sequence (22).

In this study, the c-Myc protein also becomes undetectable in tumors treated with the most effective treatment schedule. c-myc antisense oligonucleotides have been shown to increase the G<sub>2</sub>-M block elicited by DDP treatment, which results in a dual effect of irreversible block in G<sub>2</sub>-M phases and an increase of apoptotic cells (8).

A number of other recent studies have centered on the use of drugs targeted to c-myc. In fact c-myc inactivation has been shown to reverse tumorigenesis in transgenic mice, which conditionally express the oncogene in various tissues in *myc*-induced papillomas and pancreatic islet cell tumors and to induce sustained regression of *myc*-induced lymphoma, leukemias, and osteogenic sarcoma (23). Therefore, c-myc inactivation by antisense oligonucleotides after the administration of antisense oligonucleotides bcl-2 and DDP may explain the efficacy of this combination. In addition, the analysis of the AI and PI shows that the efficacy of ODN bcl-2→DDP→ODN c-myc is related to the significant increase of AI/PI ratio. Besides the inhibition of tumor mass, this effect contributes to the increase of mice survival.

Analysis of blood vessels in untreated and treated tumors showed a significant reduction of tumor vessel density in treated tumors. These results are in agreement with our previous data demonstrating the relevant role of *bcl-2* in melanoma angiogenesis (24). Furthermore, we have recently shown that a bcl-2/bcl-X<sub>L</sub> antisense oligonucleotide capable of inducing a significant bcl-2 down-regulation with a concomitant slight reduction of bcl-X<sub>L</sub>, has antiangiogenic activity *in vitro* and *in vivo* (25). In fact, these ODNs are able to inhibit endothelial cell proliferation, vascular endothelial growth factor production, and vessel formation in Matrigel plugs. Moreover, the antiangiogenic effect of ODN bcl-2, combined with antisense oligonucleotides against PIK1, was observed in MDA-MB35 xenografts (26).

The combination of these effects, including bcl-2 and c-Myc down-regulation, together with reduced vessel formation, decreased PI, and increased apoptosis, results in an increase of life span of treated mice for up to 2.5 months in both for LM and NG tumors.

In conclusion, the reported results show that in combination treatment, the sequence of drug administration is a crucial choice and suggests that the presence of the target molecule is determinant for the success of targeted molecular therapy combined with antineoplastic drugs. Further studies on a larger number of melanomas will be done to validate the role of target molecules in targeted therapy.

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